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(57) Abstract

The present invention provides immunogenic oligopeptides derived from the chemokine receptor protein for use in compositions and methods for the treatment, and prevention of inflammatory responses.

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CHEMOKINE RECEPTOR PEPTIDE VACCINES FOR TREATMENT AND PREVENTION OF DIABETES

BACKGROUND OF THE INVENTION

The present invention relates to novel compositions and methods for inhibiting inflammatory responses associated with autoimmune diseases. In particular, it relates to vaccination with peptides from the extracellular regions of chemokine receptor molecules.

Chemokines constitute a family of small molecular weight cytokines that are produced in inflammation and regulate leukocyte recruitment. These molecules are ligands for seven transmembrane G protein linked receptors that induce a signaling cascade costimulation for T cell activation in addition to participating in transendothelial migration of leukocytes (Oppenheim *et al. Ann. Rev. Immunol.* 9:617-648 (1991), Premback *et al. Nat. Med.* 2:1174-1178 (1996)). Two subfamilies of chemokines, referred to as CC and CXC, have been discovered. CC and CXC chemokines are distinct from each other in their N terminal amino acid sequence which starts either with cysteine-cysteine or cysteine-X-cysteine where X is typically another L-amino acid. They are also distinct in their binding pattern to their receptors. For example, the CC chemokines bind to CC receptors and not to CXC receptors and vice versa.

Different chemokines regulate the trafficking of distinct populations of hemopoietic cells by activating specific 7-transmembrane receptors expressed by these cells (Baggiolini *et al. Adv. Immunol.* 55:97-179 (1994); Gerard *et al. Curr. Opin. Immunol.*6:140-145 (1994)). Recent publications indicate that the Th1 and Th2 subsets of regulatory T cells are uniquely characterized by the chemokine receptors CXCR3 and CCR3, respectively (Sallusto, *et al. J. Exp. Med.* 187:875-883 (1998); Bonecchi, *et al. J. Exp. Med.* 187:129-134 (1998); Qin, *et al. J. Clin Invest.* 101:746-754 (1998)). Several studies have correlated the expression of three specific chemokines, IP-10, RANTES, and MCP-1, produced by astrocytes in the CNS with the presence of inflammatory infiltration within this tissue during the early phase of EAE (Ransohoff, *et al. FASEB J.* 7:592-600 (1993);

Glabinski *et al. Am. J. Pathol.* 150:617-630 (1995) Godiska, *et al. J. Neuroimmunol.* 58:167-176 (1995); and Eng *et al. Neurchem. Res.* 21:511-525 (1996)). While all three chemokines have been shown to be capable of recruiting T lymphocytes in certain experimental models, IP-10 has been demonstrated to be specific for this lineage of hemopoietic cells (Taub *et al. J. Exp. Med.* 177:1809-1814 (1993)); Carr, *et al. Proc. Natl. Acad. Sci. USA* 91:3652-3656 (1994); and Farber, *J. Leukoc. Biol.* 61:246-257 (1997). MBP-immunized rats intrathecaly infused with an antisense phosphorothioate oligonucleotide to crg-2 (the murine homologue of human IP-10) show reduced disease clinical score of EAE (Wojcik, *et al. J. Pharmacol. Exp. Ther.* 278:404-410 (1996)).
In addition, higher expression of some of the chemokine receptors such as CXCR3 on IL2 activated human T lymphocytes and not on resting T lymphocytes has been demonstrated (Loetscher *et al. J. Exp. Med.* 184:963-969 (1996)).

Multiple sclerosis (MS) is a T cell-dependent autoimmune disease caused by localized demyelination in the central nervous system (CNS), with only limited therapeutic options available to patients. Extensive investigation has indicated that these autoreactive T lymphocytes frequently, though not always, express the Th1 phenotype of high level production of IFNg, IL-2 and TNFa, with little to no IL-4, IL-5 and IL-10.

Current therapeutics for autoimmune diseases, such as MS, involve the use of antiinflammatory agents or general immunosuppressants. Prior art methods for controlling autoimmune disease fail to provide a simple self-mediated method for specifically eliminating inflammatory responses mediated by chemokines associated with the autoimmune responses. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods of inducing an immune response against a chemokine receptor molecule in a patient. The methods comprise administering to the patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and an immunogenic chemokine receptor polypeptide from a extracellular region of a chemokine receptor molecule, for example CXCR3.

The immunogenic peptides are preferably conformationally constrained, for example by cyclization. The length of the immunogenic peptide is not critical to the invention. Typically, the peptide consists of between about 10 and about 50 residues, more

often between about 15 and about 30 residues. Exemplary immunogenic peptides of the invention include MVLEVSDHQVLNDAEVAALL, ENFSSSYDYGENESDSCCTS, PPCPQDFSLNFDRAFLPA, DAAVQWVFGSGLCKV, SAHHDERLNATHCQYN, FPQVGRTALRVLQLVAG, and DILMDLGALARNCGRESRVDVAKS.

The immunogenic peptides can be administered by any of a number of means. Typically they are administered parenterally. The adjuvant can be, for example, alum.

In preferred embodiments, the method are used to inhibit recruitment of T cells to inflammation sites in a patient. Typically the inflammatory response is associated with an autoimmune disease, such as multiple sclerosis.

The invention also provides pharmaceutical compositions suitable for use in the above methods.

Definitions

The term "peptide" is used interchangeably with "oligopeptide" or "polypeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the α-amino and carbonyl groups of adjacent amino acids.

The term "cyclic peptide" refers to peptides in which the N-terminal residue is linked to the C-terminal residue either directly or through an intermediate. Examples of links between the two residues include disulfide bonds and thioether linkages as described below.

An "immunogenic chemokine receptor polypeptide" of the present invention is a polypeptide capable of eliciting an immune response against a chemokine receptor molecule associated with inflammation in autoimmune responses in a patient, such as multiple sclerosis. As set forth in more detail below, the sequence of residues in the polypeptide will be identical to or substantially identical to a polypeptide sequence in a chemokine receptor molecule. Thus, a polypeptide of the invention that has a sequence "from a extracellular region of a chemokine receptor molecule" is polypeptide that has a sequence either identical to or substantially identical to the naturally occurring chemokine receptor amino acid sequence of the region.

As used herein a "extracellular region" of a chemokine receptor molecule is a region of the molecule which is exposed on the surface of a cell expressing the native molecule. Figure 1 provides a schematic of the extracellular domains of the human CXCR3

molecule. This molecule has four extracellular regions designated as SP-1, SP-2, SP-3 and SP-4, starting from the N-terminus.

A "non-specific immune response enhancer" is any substance that enhances an immune response to an exogenous antigen. Examples of non-specific immune response enhancers include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into which the compound is incorporated; *see e.g.*, Fullerton, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995).

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the chemokine receptor polypeptides of this invention do not contain materials normally associated with their *in situ* environment, *e.g.*, other surface proteins on T cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated polypeptides of this invention do not contain such endogenous co-purified protein.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in a oligopeptide by an amide bond or amide bond mimetic.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in

conservative substitutions. the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch. thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, e.g., the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to sequences or subsequences that have at least 60%, preferably 70%, more preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence, which has substantial sequence or subsequence complementarity when the test sequence has substantial identity to a reference sequence.

One of skill in the art will recognize that two polypeptides can also be "substantially identical" if the two polypeptides are immunologically similar. Thus, overall protein structure may be similar while the primary structure of the two polypeptides display significant variation. Therefore a method to measure whether two polypeptides are substantially identical involves measuring the binding of monoclonal or polyclonal antibodies to each polypeptide. Two polypeptides are substantially identical if the antibodies specific for a first polypeptide bind to a second polypeptide with an affinity of at least one third of the affinity for the first polypeptide.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally*, *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation

(E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes

every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, highly stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. Lower stringency conditions are generally selected to be about 15-30 °C below the T_m. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 time background hybridization.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

In the present invention, genomic DNA or cDNA comprising nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed here. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of

ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, an RNA gel or DNA gel blot hybridization analysis.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows acidification rate changes due to the binding of anti-human CXCR3 Mab or IP-10 to CXCR3-NSO transfectants. $5X10^5$ CXCR3-NSO transfectant cells and untransfected "BONZO-NSO" cells (negative control) were spotted in the microphysiometer chambers along with agarose and 1, 5, 10 mglml anti-CXCR3 (Figure 1a) or IP-10 (Figure 1b) was pumped for 10 min. There is a dose dependent increase in the acidification rates with anti-CXCR3 in NSO-CXCR3 transfectant cells. Untransfected BONZO-NSO cells did not show any change in acidification rate changes either with the anti-CXCR3 antibody, or IP-10. The arrow indicates the time that ligands were added to the cells.

Figure 2 shows the structure of the seven transmembrane G-protein coupled human CXCR3. Only amino acids in the extracellular domains are given

Figure 3 is a schematic of the experiments in which peptides vaccines of the

invention were used to prevent EAE in mice.

Figure 4 shows the results of experiments in which peptides vaccines of the invention were used to prevent EAE in mice.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides immunogenic polypeptides derived from chemokine receptor protein sequences for use in compositions and methods for the treatment and prevention of inflammatory responses. The polypeptides are capable of inducing an immune response against chemokine receptor molecules which mediate inflammatory responses associated with various diseases. In preferred embodiments the polypeptides of the invention are conformationally constrained (*e.g.*, cyclized) and derived from extracellular regions of human CXCR3. Immunization with the polypeptides of the invention provides a specific immune response to particular chemokine receptors and results in the specific inhibition of the inflammatory responses mediated by these molecules.

The methods can be used to treat any inflammatory response mediated by chemokines. In particular, the methods are useful for treatment of autoimmune diseases, such as multiple sclerosis. Multiple sclerosis (MS), a human demyelinating disease which afflicts 600,000 individuals worldwide, results from damage of the myelin sheath of oligodendroglial cells in the Central Nervous System (CNS). Although the pathogenesis and etiology of MS have not yet been established, it is widely believed that the disease has an immunological basis and that both genetic and environmental factors make a contribution to the pathogenesis. The central mediator of autoimmune attack is believed to be host CD4+ T cells specific for one or more autoantigens in the CNS, with subsequent production of an array of tissue-destructive inflammatory mediators following autoantigen-activation of these cells. Indeed, immunohistochemical analysis of the focal plaques of demyelination which occur in the brains of MS patients as a consequence of MS pathology have revealed the presence of CD4+ T cells infiltrating these plaques.

Improved understanding of the immunopathological mechanisms underlying MS has developed from the study of experimental models of demyelination. The most commonly used model, experimental allergic encephalomyelitis (EAE), is an autoimmune inflammatory disorder of genetically susceptible mice that is mediated by autoantigen-specific CD4+ MHC class II restricted T cells. In susceptible SJL/J mice, the

disease can display a relapsing-remitting clinical course of paralysis. which makes it an ideal system to study the efficacy of various immunoregulatory strategies both in the prevention and treatment of disease.

The current invention is focused not on the cytokine-producing phenotype of autoreactive T cells in this disease setting, but on their trafficking from the host circulation to the site of pathology, for example within the CNS in the case of MS. As noted above, hemopoietic cell migration is regulated by chemokines.

In some embodiments, the invention provides peptide vaccines against the 7-transmembrane chemokine receptor designated CXCR3. Consistent with the chemotactic fingerprint of IP-10, CXCR3 is expressed exclusively on activated effector T lymphocytes. Exemplary peptides of the invention include peptides derived from the extracellular domains of the CXCR3 protein are presented in Table 1.

In preferred embodiments, the peptides of the invention are cyclized. Methods for cyclizing peptides are described in detail below. In those cases in which the peptides are cyclized by disulfide linkages, one of skill will recognize that the peptides will further comprise cysteine residues either within the peptide or at each terminus.

Polypeptides suitable for use in the present invention can be obtained in a variety of ways. Conveniently, they can be synthesized by conventional techniques employing automatic synthesizers, such as the Beckman, Applied Biosystems, or other commonly available peptide synthesizers using well known protocols. They can also be synthesized manually using techniques well known in the art. See, e.g. Stewart and Young, *Solid Phase Peptide Synthesis*, (Rockford, Ill., Pierce), 2d Ed. (1984).

Alternatively, DNA sequences which encode the particular chemokine receptor polypeptide may be cloned and expressed to provide the peptide. Nucleic acid molecules encoding chemokine receptors are known in the art and sequences of such genes are available, for instance, from GenBank (*see, e.g.*, GenBank Accession Nos. HSU83326 HSU97123, and AF005058).

Standard techniques can be used to screen cDNA libraries to identify sequences encoding the desired sequences (see. Sambrook et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Fusion proteins (those consisting of all or part of the amino acid sequences of two or more proteins) can be recombinantly produced. In addition, using *in vitro* mutagenesis techniques,

unrelated proteins can be mutated to comprise the appropriate sequences.

Chemokine receptor proteins from a variety of natural sources are also conveniently isolated using standard protein purification techniques. Peptides can be purified by any of a variety of known techniques, including, for example, reverse phase high-performance liquid chromatography (HPLC), ion-exchange or immunoaffinity chromatography, separation by size, or electrophoresis (See, generally, Scopes, R., *Protein Purification*, Springer-Verlag, N.Y. (1982)).

It will be understood that the immunogenic chemokine receptor polypeptides of the present invention may be modified to provide a variety of desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide. For instance, the peptides can be modified by extending, decreasing the amino acid sequence of the peptide. Substitutions with different amino acids or amino acid mimetics can also be made.

The peptides employed in the subject invention need not be identical to peptides disclosed in the Example section, below, so long as the subject peptides are able to induce an immune response against the desired chemokine receptor molecule. Thus, one of skill will recognize that a number of conservative substitutions (described in more detail below) can be made without substantially affecting the activity of the peptide.

Single amino acid substitutions, deletions, or insertions can be used to determine which residues are relatively insensitive to modification. Substitutions are preferably made with small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues. The effect of single amino acid substitutions may also be probed using D-amino acids. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased immunogenicity may also be achieved by such substitutions, compared to the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

The substituting amino acids, however, need not be limited to those naturally occurring in proteins, such as L-α-amino acids, or their D-isomers. The peptides may be substituted with a variety of moieties such as amino acid mimetics well known to those of skill in the art.

The individual residues of the immunogenic chemokine receptor polypeptides can be incorporated in the peptide by a peptide bond or peptide bond mimetic. A peptide bond mimetic of the invention includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. VII (Weinstein ed., 1983). Several peptide backbone modifications are known, these include, $\psi[CH_2S]$, $\psi[CH_2NH]$, $\psi[CSNH_2]$, $\psi[NHCO]$, $\psi[COCH_2]$ and $\psi[(E)$ or (Z) $CH{=}CH]$. The nomenclature used above, follows that suggested by Spatola, above. In this context, ψ indicates the absence of an amide bond. The structure that replaces the amide group is specified within the brackets.

Amino acid mimetics may also be incorporated in the peptides. An "amino acid mimetic" as used here is a moiety other than a naturally occurring amino acid that conformationally and functionally serves as a substitute for an amino acid in a polypeptide of the present invention. Such a moiety serves as a substitute for an amino acid residue if it does not interfere with the ability of the peptide to illicit an immune response against the appropriate chemokine receptor molecule. Amino acid mimetics may include non-protein amino acids, such as β-γ-δ-amino acids, β-γ-δ-imino acids (such as piperidine-4-carboxylic acid) as well as many derivatives of L-α-amino acids. A number of suitable amino acid mimetics are known to the skilled artisan, they include cyclohexylalanine, 3-cyclohexylpropionic acid, L-adamantyl alanine, adamantylacetic acid and the like. Peptide mimetics suitable for peptides of the present invention are discussed by Morgan and Gainor, (1989) *Ann. Repts. Med. Chem.* 24:243-252/

As noted above, the peptides employed in the subject invention need not be identical, but may be substantially identical, to the corresponding sequence of the target chemokine receptor molecule. Therefore, the peptides may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. The polypeptides of the invention can be modified in a number of ways so long as they comprise a sequence substantially identical (as defined below) to a sequence in the target region of the chemokine receptor molecule.

Alignment and comparison of relatively short amino acid sequences (less than

about 30 residues) is typically straightforward. Comparison of longer sequences may require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the methods described above.

In some embodiments, the polypeptides of the invention may be fusion proteins that comprise multiple polypeptides as described herein, or that comprise at least one polypeptide as described herein and an unrelated sequence. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the protein or to enable the protein to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the protein.

Fusion proteins may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion protein is expressed as a recombinant protein, allowing the production of increased levels, relative to a non-fused protein, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion protein that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral

amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*., *Gene 40*:39-46, 1985; Murphy *et al.* *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The polypeptides of the invention typically comprise at least about 10 residues and more preferably at least about 15 residues from the extracellular domain of a chemokine receptor. In certain embodiments the peptides will not exceed about 50 residues and typically will not exceed about 30 residues. For instance, the peptides described below consist of about 15 to about 25 residues.

In the preferred embodiments of the invention, the immunogenic peptides are conformationally constrained. Means for achieving this are well known in the art (*see, e.g.*, Hruby and Bonner in *Methods in Molecular Biology, Volume 35: Peptide Synthesis Protocols* Pennington and Dunn eds (Humana Press, Totowa NJ, 1994). A preferred means for preparing conformationally constrained peptides is through cyclization. Any method commonly used to produce cyclized oligopeptides can be used to produce the peptides of the invention. For example, in certain embodiments the peptides will include cysteine residues at both termini, which allow the production of cyclic peptides through disulfide linkages. Treatment of a such a peptide with an oxidizing agent such as oxygen, iodine or similar agent will produce a cyclic peptide which may be further purified using chromatographic or other methods of chemical purification. Construction of cyclic peptides can also be accomplished through thioether linkages. For instance, N-bromoacetyl-derivatized peptides can be reacted with sulfhydryl-containing residues, such as cysteine. Cyclization occurs by reaction of the free sulfhydryl of cysteine in the peptide with the bromoacetyl group to form a thioether linkage (Robey *et al.*, *Anal. Biochem.* 177:373-7 (1989) and U.S. Patent No. 5,066,716).

Other methods of constructing cyclic peptides are known to those skilled in the art. These include side chain-side chain, side chain-main chain and main chain-main chain cyclizations. In addition, linkers can be used to join the amino and carboxyl termini of a peptide. The linker is capable of forming covalent bonds to both the amino and carboxyl

terminus. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. The linkers may be joined to the carboxyl and amino terminal amino acids through their side groups (e.g., through a disulfide linkage to cysteine) or through the alpha carbon amino and carboxyl groups of the terminal amino acids.

For a general discussion of suitable methods for cyclization *see*, Hruby and Bonner in *Methods in Molecular Biology, Volume 35: Peptide Synthesis Protocols* Pennington and Dunn eds (Humana Press, Totowa NJ, 1994). For instance, cyclizations may include formation of carba analogs and thioethers (Lebl *et al.* in *Peptides 1986* Proceedings of the 19th European Peptide Symposium pp. 341-344; Robey *et al.*, *Anal. Biochem.* 177:373-7 (1989) and U.S. Patent No. 5,066,716), bis-thioethers (Mosberg *et al. JACS* 107:2986-2987 (1985)), azopeptides (Siemion *et al. Mol. Cell. Biochem.* 34: (1991)), and other cyclic structures, such as bridging structures (Charpentier, M., *et al.*, *J. Med. Chem.* 32(6):1184-1190 (1989), Thaisrivongs, S., *et al.*, *J. Med. Chem.* 34(4):127 (1991) and Ozeki, E., *et al.*, *Int. J. Peptide Protein Res.* 34:111 (1989)). Cyclization from backbone-to-backbone positions may also be used.

Bridging is a special type of cyclization in which distant sites in a peptide are brought together using separate bridging molecules or fragments. Bridging molecules may include, for example, succinic anhydride molecules (Charpentier, B., *et al.*, *supra*), and carboxymethylene fragments (Thaisrivongs, S., *et al.*, *supra*). Bridging by metals can also be used (Ozeki, E., *et al.*, *supra*).

In some embodiments, the peptides include two or more cystine residues. The cystines can be substituted or added within the peptide or at either terminus. The position of the cystines is not critical so long as disulfide linkages can form between them which allow the production of cyclic peptides. For example, treatment of such a peptide with an oxidizing agent such as oxygen, iodine or similar agent will produce a cyclic peptide which may be further purified using chromatographic or other methods of chemical purification.

In addition to use of peptides, antibodies raised against peptides of the invention can be used to inhibit inflammatory responses. Antibodies can be raised to the peptides of the present invention using techniques well known to those of skill in the art. Anti-idiotypic antibodies can also be generated. The following discussion is presented as a

general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

A number of immunogens can be used to produce antibodies specifically reactive with the peptides. For instance, the entire chemokine receptor molecule or fragments containing the desired sequence can be used. Synthetic peptides as disclosed here can be used either in linear form or cyclized.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemanocyanin, *etc.*), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (*see, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate (preferably mammalian) host. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about .1 mM, more usually at least about 50 μM, and most preferably at least about 1 μM or better.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g.*, Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546; and Vaughan *et al.* (1996) *Nature Biotechnology*, 14: 309-314).

Frequently, the peptides and antibodies of the invention will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

The antibodies of this invention can also be administered to an organism (*e.g.*, a human patient) for therapeutic purposes (*e.g.*, to inhibit an autoimmune response). Antibodies administered to an organism other than the species in which they are raised are often immunogenic. Thus, for example, murine antibodies administered to a human often induce an immunologic response against the antibody (*e.g.*, the human anti-mouse antibody (HAMA) response) on multiple administrations. The immunogenic properties of the antibody are reduced by altering portions, or all, of the antibody into characteristically human sequences thereby producing chimeric or human antibodies, respectively.

Chimeric antibodies are immunoglobulin molecules comprising a human and non-human portion. More specifically, the antigen combining region (or variable region) of a

humanized chimeric antibody is derived from a non-human source (*e.g.*, murine) and the constant region of the chimeric antibody (which confers biological effector function to the immunoglobulin) is derived from a human source. The chimeric antibody should have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369). An alternative approach is the generation of humanized antibodies by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. *See* Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861.

In one preferred embodiment, recombinant DNA vector is used to transfect a cell line that produces an antibody against a peptide of the invention. The novel recombinant DNA vector contains a "replacement gene" to replace all or a portion of the gene encoding the immunoglobulin constant region in the cell line (*e.g.*, a replacement gene may encode all or a portion of a constant region of a human immunoglobulin, or a specific immunoglobulin class), and a "target sequence" which allows for targeted homologous recombination with immunoglobulin sequences within the antibody producing cell.

In another embodiment, a recombinant DNA vector is used to transfect a cell line that produces an antibody having a desired effector function, (*e.g.*, a constant region of a human immunoglobulin) in which case, the replacement gene contained in the recombinant vector may encode all or a portion of a region of an antibody and the target sequence contained in the recombinant vector allows for homologous recombination and targeted gene modification within the antibody producing cell. In either embodiment, when only a portion of the variable or constant region is replaced, the resulting chimeric antibody may define the same antigen and/or have the same effector function yet be altered or improved so that the chimeric antibody may demonstrate a greater antigen specificity, greater affinity binding constant, increased effector function, or increased secretion and production by the transfected antibody producing cell line, *etc.*

In another embodiment, this invention provides for fully human antibodies. Human antibodies consist entirely of characteristically human polypeptide sequences. The

human antibodies of this invention can be produced in using a wide variety of methods (*see, e.g.*, Larrick *et al.*, U.S. Pat. No. 5,001,065). In one preferred embodiment, the human antibodies of the present invention are produced initially in trioma cells. Genes encoding the antibodies are then cloned and expressed in other cells, particularly, nonhuman mammalian cells. The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983), *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. The antibody-producing cell lines obtained by this method are called triomas because they are descended from three cells; two human and one mouse. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

Formulation and Administration

The peptides or antibodies (typically monoclonal antibodies) of the present invention and pharmaceutical compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent deleterious immune inflammatory responses, particularly those associated with autoimmune responses. Over 30 autoimmune diseases are presently known, including myasthenia gravis (MG), multiple sclerosis (MS), systemic lupus erythematosis (SLE), rheumatoid arthritis (RA), insulin-dependent diabetes mellitus (IDDM), and the like. Suitable formulations are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985).

The immunogenic peptides or antibodies of the invention are administered prophylactically or to an individual already suffering from the disease. The peptide compositions are administered to a patient in an amount sufficient to elicit an effective immune response to the chemokine receptor molecule from which the peptides are derived. An effective immune response is one that inhibits recruitment of T cells to sites of inflammation. An amount adequate to accomplish this is defined as "therapeutically effective dose" or "immunogenically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 0.1 mg to about 1.0 mg per 70 kilogram patient, more commonly from about 0.5 mg to about 0.75 mg per 70 kg of

body weight. Boosting dosages are typically from about 0.1 mg to about 0.5 mg of peptide using a boosting regimen over weeks to months depending upon the patient's response and condition. A suitable protocol would include injection at time 0, 4, 2, 6, 10 and 14 weeks, followed by further booster injections at 24 and 28 weeks.

It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of autoimmune disease. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In some circumstances, loading doses followed by boosting doses may be required. The resulting immune response helps to cure or at least partially arrest symptoms and/or complications. Vaccine compositions containing the peptides are administered prophylactically to a patient susceptible to or otherwise at risk of the disease to elicit an immune response against the target Chemokine receptor antigen.

The pharmaceutical compositions (containing either peptides or antibodies) are intended for parenteral or oral administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., subcutaneously, intradermally, or intramuscularly. Thus. the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline. 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

As noted above, the peptide compositions are intended to induce an immune response to the peptides. Thus, compositions and methods of administration suitable for maximizing the immune response are preferred. For instance, peptides may be introduced into a host, including humans, linked to a carrier or as a homopolymer or heteropolymer of active peptide units. Alternatively, the a "cocktail" of polypeptides can be used. A mixture of more than one polypeptide has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies to a number of epitopes. For instance, polypeptides comprising sequences from extracellular regions of α and β chains may be used in combination. Useful carriers are well known in the art, and include, e.g., KLH, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like.

The use of more than one polypeptide is particularly useful to enhance the immune response against polypeptides of the invention. As demonstrated below, although the polypeptides may be derived from self Chemokine receptor molecules expressed in the patient, they can induce an immune response. In some instances, the immune response to the self polypeptide may not be sufficiently strong. In these instances, it may be necessary to break tolerance to the polypeptide. The compositions may comprise one or more of the foreign polypeptides that are sufficiently similar to the self polypeptides to induce an immune response against both the foreign and self polypeptides (*see*, Mamula *et al. J. Immunol.* 149:789-795 (1992). Suitable proteins include synthetic polypeptides designed for this purpose or polypeptide sequences from homologous proteins from natural sources, such as proteins encoded by a different allele at the same locus as the self polypeptide.

Any of a variety of non-specific immune response enhancers may be employed in the vaccines of this invention. For example, an adjuvant may be included.

Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants. Other suitable adjuvants include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-Lalanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against the immunogenic peptide.

A particularly useful adjuvant and immunization schedule are described in Kwak et al. *New Eng. J. Med.* 327-1209-1215 (1992). The immunological adjuvant described there comprises 5% (wt/vol) squalene, 2.5% Pluronic L121 polymer and 0.2 % polysorbate in phosphate buffered saline.

The concentration of immunogenic peptides of the invention in the pharmaceutical formulations can vary widely, i.e. from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into a host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits an immune response. Vaccinia vectors and methods useful in immunization protocols

are described in, e.g., U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., *Salmonella typhi* vectors and the like, will be apparent to those skilled in the art from the description herein.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems 15*:143-198, 1998, and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; Kolls et al., *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; and Guzman et al., *Cir. Res. 73*:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993 (*see, also* Wolff *et. al.*, *Science* 247: 1465-1468 (1990) as well as U.S. Patent Nos. 5,580,859 and 5,589,466). The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In order to enhance serum half-life, the peptides may also be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional techniques may be employed which provide an extended serum half-life of the peptides. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028.

The peptides or antibodies of the invention can also be used for diagnostic purposes. For instance, peptides can be used to screen for autoantibodies to ensure that the vaccination has been effective. Antibodies can be used to detect the presence of particular Chemokine receptor molecules associated with disease.

The following examples are offered by way of illustration, not by way of limitation.

Example 1

This example describes a reproducible bioassay for CXCR3 activation.

A stable line transfectant of NSO-1 cells expressing the cDNA for human CXCR3 was prepared according to standard techniques. The surface expression of hu-CXCR3 on the transfectants compared to untransfected parent NSO-1 cells was confirmed by FACS staining using mouse anti-human CXCR3 monoclonal antibody (R&D systems).

To establish a bioassay for CXCR3 activation, the CXCR3 transfectant cells were cultured with either human IP-10, or the mouse anti-human CXCR3 antibody, and the physiological response of the cells was measured using a microphysiometer. This machine measures changes in the pH of the extracellular medium of the cell cultures which result from ligand receptor binding on the cell surface. These extracellular acidification rate measurements have previously been used as markers of antigen specific T cell activation and T cell epitope identification. The current assay is used to provide a biological read out for the identification of chemokine or chemokine receptor peptides involved in binding to IP-10 or agonistic anti-CXCR3 antibody.

The experiments indicated that human IP-10 and anti-CXCR3 antibody both triggered substantial acidification rate changes in the CXCR3 transfectants, as shown in

Figure 1. Importantly, the same ligands induce no change in acidification rate of untransfected NSO-1 cells (Fig. 1).

Example 2

This example describes synthesis of human CXCR3 derived peptides.

A schematic representation of the surface portion of human CXCR3 is given in Figure 2. The receptor has 4 surface portions, 4 intracellular portions and seven transmembrane portions. The surface portions were designated starting from N-terminus as SP-1, SP-2, SP-3 and SP-4.

The peptides shown in Table 1 derived from these four surface portions were prepared by solid phase peptide synthesis. The names of the peptides are based on the surface portion of CXCR3 from which they were derived. For example, SP-1-1 means this peptide was derived from the first portion of the CXCR3 protein.

Table 1.

Peptide Name	Sequence
SP-1-1	MVLEVSDHQVLNDAEVAALL -NH2
SP-1-2	ENFSSSYDYGENESDSCCTS -NH2
SP-1-3	PPCPQDFSLNFDRAFLPA -NH2
SP-2-1	DAAVQWVFGSGLCKV -NH2
SP-3-1	SAHHDERLNATHCQYN -NH2
SP-3-2	FPQVGRTALRVLQLVAG -NH2
SP-4-1	DILMDLGALARNCGRESRVDVAKS -NH2

The ability of these receptor-derived peptides to bind anti-CXCR3 antibody was evaluated using a standard ELISA format. The peptides were dissolved in 0.1 M bicarbonate buffer and coated on a 96 well ELISA plate overnight. The excess peptides were removed and nonspecific binding sites in the wells were blocked by 0.1 % bovine serum albumin. Anti-CXCR3 antibody (0.5 ug/well) was added to these wells and incubated for 2 hours. Excess antibody was removed by washing with PBS. HRP conjugated goat anti-mouse antibody was used as secondary antibody for detection. Two of the seven receptor-derived peptides, namely SP-1-3 and SP-4-1, showed substantial binding to anti-CXCR3 antibody. Further support for the conclusion that anti-CXCR3 antibody bound

two of the seven receptor-derived peptides was provided by FACS analysis of the same interactions. These studies revealed that the receptor-derived peptide SP-4-1 potently blocked binding of anti-CXCR3 antibody to the CXCR3 cell line transfectant. The receptor-derived peptide SP-1-3 provided partial inhibition of the binding of the antibody to CXCR3 transfectants. In contrast, an antibody nonbinding receptor-derived peptide, SP-2-1, failed to inhibit binding to CXCR3 transfectants. The data described here collectively demonstrate that anti-CXCR3 antibody can bind to two separate peptide portions of the CXCR3 extracellular domain.

Example 3

This example demonstrates that peptide vaccines of the invention can be used to prevent EAE in mice.

<u>Protocols for animal experiments:</u>

SJL mice (6-8 weeks old) were obtained from Jackson Laboratories. They were kept in quarantine for two weeks. These mice get EAE when immunized with a peptide from proteolipid protein (PLP). The peptide sequence used for the immunization of these mice is PLP 139-151 and is amidated at the C terminus (HSLGWLGHPDKF-NH2). For the experiments, induction of the disease is considered Day 0. Three weeks before the induction of the disease, the mice were vaccinated with Human CXCR3S-P4-1 peptide mixed with complete Freund's Adjuvant.

<u>Preparation of peptide CFA emulsion for treatment</u>

4mg of human CXCR3 SP-4-1 peptide was dissolved in 1 ml of phosphate buffered saline pH 7.4 (PBS). One ml of CFA obtained from VWR (Difco, Adjuvant Complete H37RA) was added to the peptide solution and the mixture was sonicated for 5 seconds using a fine tip sonicator. The emulsion was taken in 1 ml syringe (needed 2 syringes) fitted with a 25 gauge needle. Each mouse was given 100 ul of the emulsin under each flank near the hind legs by subcutaneous injection (total volume per mouse = 200 ul, total peptide per mouse = 200 ug).

<u>Preparation of CFA alone:</u>

One ml of PBS and 1 ml of CFA were mixed and sonicated for 5 seconds and the emulsin is drawn into a 1 ml syringe fitted with a 25 gauge needle. Each mouse was given 100 ul of this emulsion under each flank near hind legs by subcutaneous injection (total volume of CFA per mouse = 200 ul).

Induction of Disease:

To an aqueous solution of peptide (4 mg per ml in PBS) equal volume of CFA was added and the mixture was sonicated for 5 seconds. The emulsion was drawn in a 1 ml syringe fitted with a 25 gauge needle and subcutaneously injected (200 ul total per mouse) in mice at the foot pad and back.

Testing the antibody response to SP4-1 peptide

The mice were bled at week 0 and week 5 and serum was tested by ELISA for the presence of antibodies against the human CXCR3 SP-4-1 peptide. Briefly, the SP4-1 peptide dissolved in 0.1 M sodium bicarbonate buffer was plated on a 96-well ELISA plate overnight. The nonspecific binding sites on the plate were coated with 0.1% bovine serum albumin solution in PBS. The wells were washed and serum (diluted in PBS) was added to the wells and incubated at room temperature for 1.5 hours. The wells were then washed and HRP conjugated anti-mouse immunoglobulin antibody was used to detect the presence of anti-SP4-1 antibodies). The results of this ELISA clearly indicate that the SP4-1 treated mice show an antibody response against this peptide.

Results of EAE study

Sp4-1 was administered with CFA at 21 days and 14 days before induction of EAE as described above (Figure 3). The results are shown in Figure 4. There it can be seen that while most of the untreated and CFA treated mice showed clinical symptoms of EAE, 7 out of 8 mice treated with SP4-1 in CFA showed no clinical symptoms.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and

are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1 A method of inducing an immune response against a chemokine receptor molecule in a patient, the method comprising administering to the patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and an immunogenic chemokine receptor polypeptide from a extracellular region of a chemokine receptor molecule.

2 The method of claim 1, wherein the chemokine receptor is CXCR3.

3 The method of claim 1, wherein the immunogenic peptide is conformationally constrained.

4 The method of claim 1, wherein the immunogenic peptide is cyclized.

5 The method of claim 1, wherein the immunogenic chemokine receptor peptide consists of between about 10 and about 50 residues.

6 The method of claim 1, wherein the immunogenic chemokine receptor peptide consists of between about 15 and about 30 residues.

7 The method of claim 1, wherein the immunogenic chemokine receptor polypeptide has an amino acid sequence selected from the group consisting of MVLEVSDHQVLNDAEVAALL, ENFSSSYDYGENESDSCCTS, PPCPQDFSLNFDRAFLPA, DAAVQWVFGSGLCKV, SAHHDERLNATHCQYN, FPQVGRTALRVLQLVAG, and DILMDLGALARNCGRESRVDVAKS.

8 The method of claim 1, wherein the administration is parenteral.

9 The method of claim 1. wherein the adjuvant is alum.

10 A method of inhibiting recruitment of T cells to inflammation site in a patient, the method comprising administering to the patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and an immunogenic chemokine receptor peptide from a extracellular region of a chemokine receptor molecule.

11 The method of claim 10, wherein the chemokine receptor is CXCR3.

12 The method of claim 10, wherein the immunogenic peptide is conformationally constrained.

13 The method of claim 10, wherein the immunogenic peptide is cyclized.

14 The method of claim 10, wherein the immunogenic chemokine receptor peptide consists of between about 10 and about 50 residues.

15 The method of claim 10, wherein the immunogenic chemokine receptor peptide consists of between about 15 and about 30 residues.

16 The method of claim 10, wherein the immunogenic chemokine receptor polypeptide has an amino acid sequence selected from the group consisting of MVLEVSDHQVLNDAEVAALL, ENFSSSYDYGENESDSCCTS, PPCPQDFSLNFDRAFLPA, DAAVQWVFGSGLCKV, SAHHDERLNATHCQYN, FPQVGRTALRVLQLVAG, and DILMDLGALARNCGRESRVDVAKS.

17 The method of claim 10, wherein the inflammatory response is associated with multiple sclerosis.

18 A pharmaceutical composition comprising an adjuvant and an isolated immunogenic chemokine receptor polypeptide from a extracellular region of a chemokine receptor molecule.

19 The composition of claim 18, wherein the chemokine receptor is CXCR3.

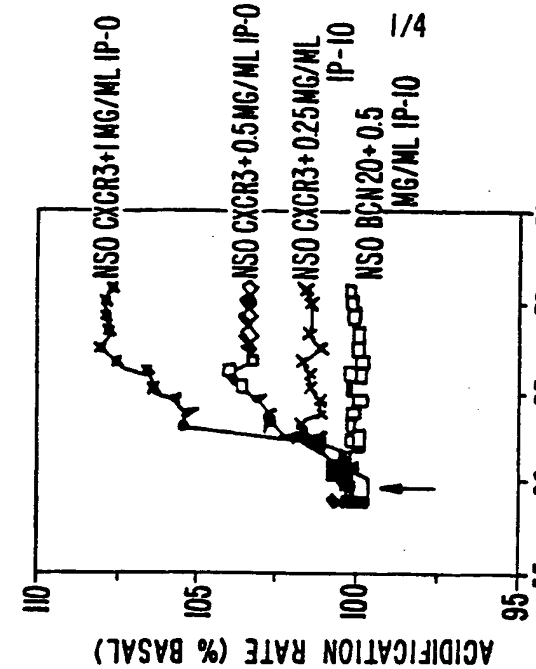
20 The composition of claim 18, wherein the immunogenic peptide is conformationally constrained.

21 The composition of claim 18, wherein the immunogenic peptide is cyclized.

22 The composition of claim 18, wherein the immunogenic chemokine receptor peptide consists of between about 10 and about 50 residues.

23 The composition of claim 18, wherein the immunogenic chemokine receptor peptide consists of between about 15 and about 30 residues.

24 The composition of claim 18, wherein the immunogenic chemokine receptor polypeptide has an amino acid sequence selected from the group consisting of MVLEVSDHQVLNDAEVAALL, ENFSSSYDYGENESDSCCTS, PPCPQDFSLNFDRAFLPA, DAAVQWVFGSGLCKV, SAHHDERLNATHCQYN, FPQVGRTALRVLQLVAG, and DILMDLGALARNCGRESRVDVAKS.


ACIDIFICATION RATE (% BASAL)
110
105
100
95
55
60
65
70
75
TIME (MIN)
NSO CXCR3+1 MG/ML IP-10
NSO CXCR3+0.5 MG/ML IP-10
NSO CXCR3+0.25 MG/ML IP-10
NSO BCN20+0.5 MG/ML IP-10

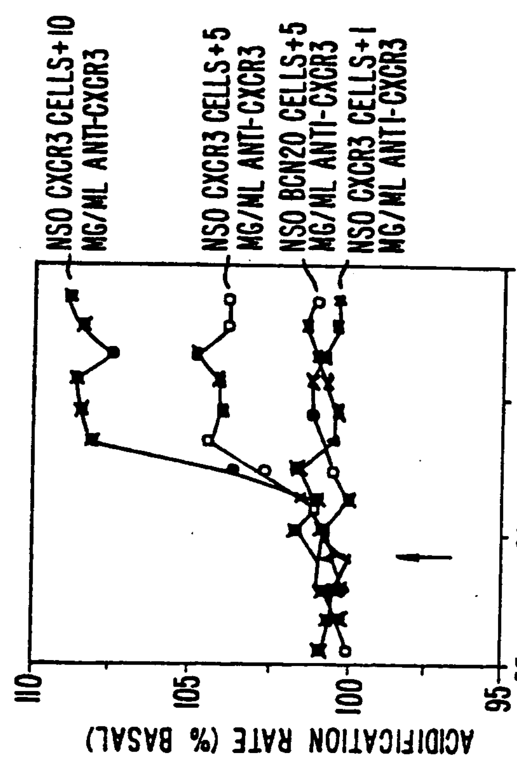

ACIDIFICATION RATE (% BASAL)
110
105
100
95
55
60
65
70
TIME (MIN)
NSO CXCR3 CELLS+10 MG/ML ANTI-CXCR3
NSO CXCR3 CELLS+5 MG/ML ANTI-CXCR3
NSO BCN20 CELLS+5 MG/ML ANTI-CXCR3
NSO CXCR3 CELLS+1 MG/ML ANTI-CXCR3

FIG. 1.

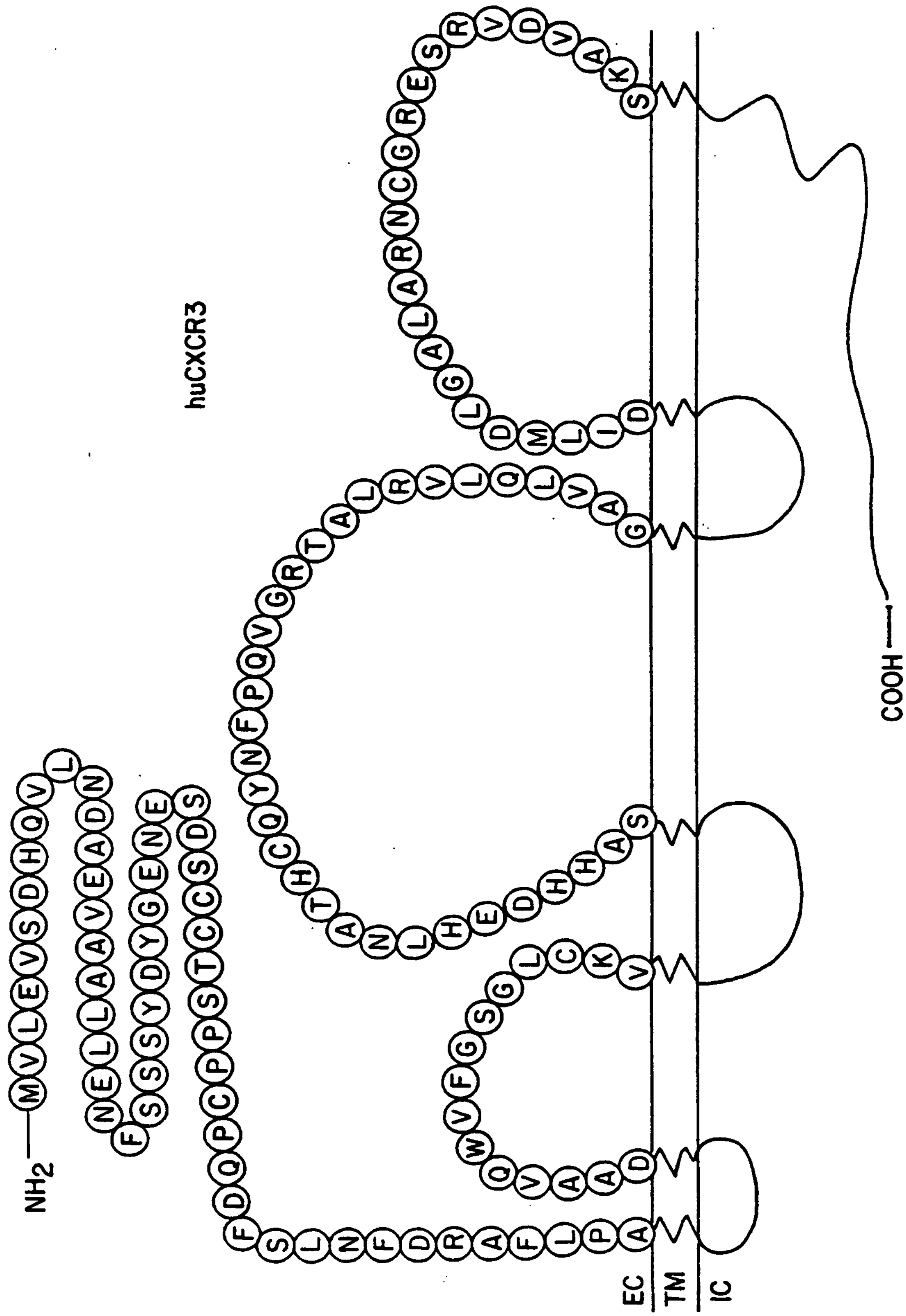


FIG. 2.

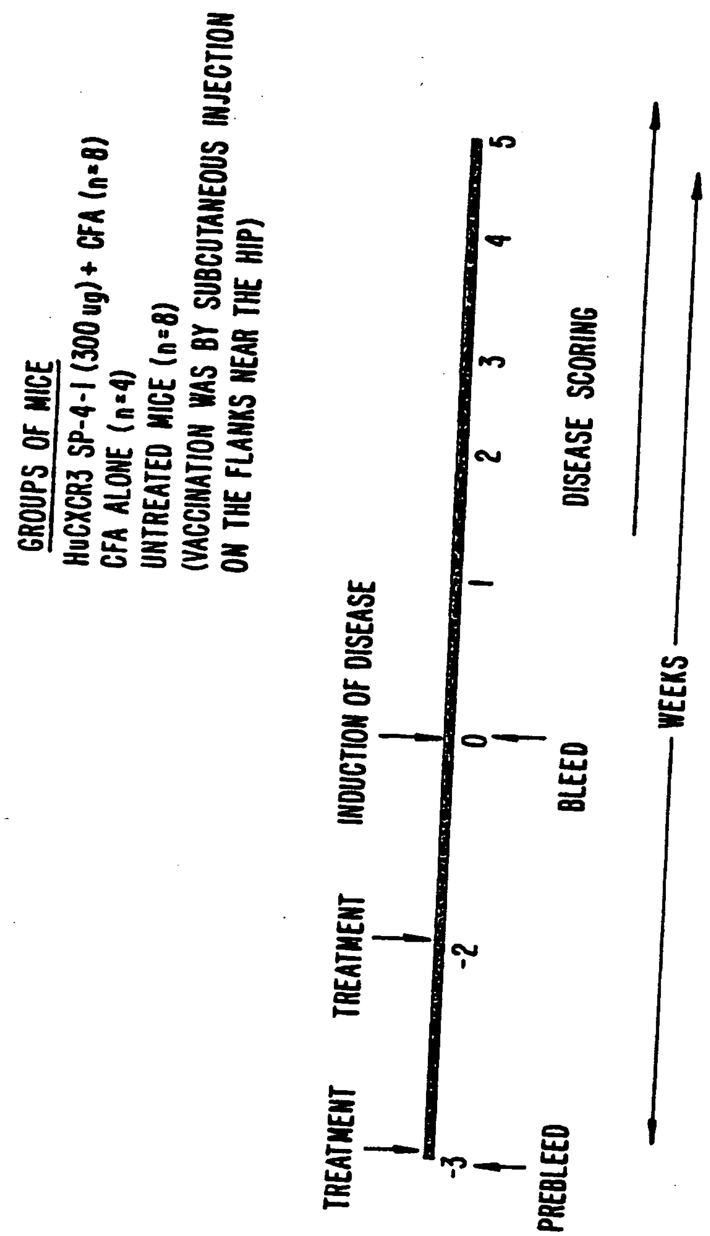

GROUPS OF MICE
HuCXCR3 SP-4-1 (300 ug) + CFA (n=8)
CFA ALONE (n=4)
UNTREATED MICE (n=8)
(VACCINATION WAS BY SUBCUTANEOUS INJECTION
ON THE FLANKS NEAR THE HIP)
TREATMENT
TREATMENT
INDUCTION OF DISEASE
-3
-2
0
1
2
3
4
5
PREBLEED
BLEED
DISEASE SCORING
WEEKS

FIG. 3.

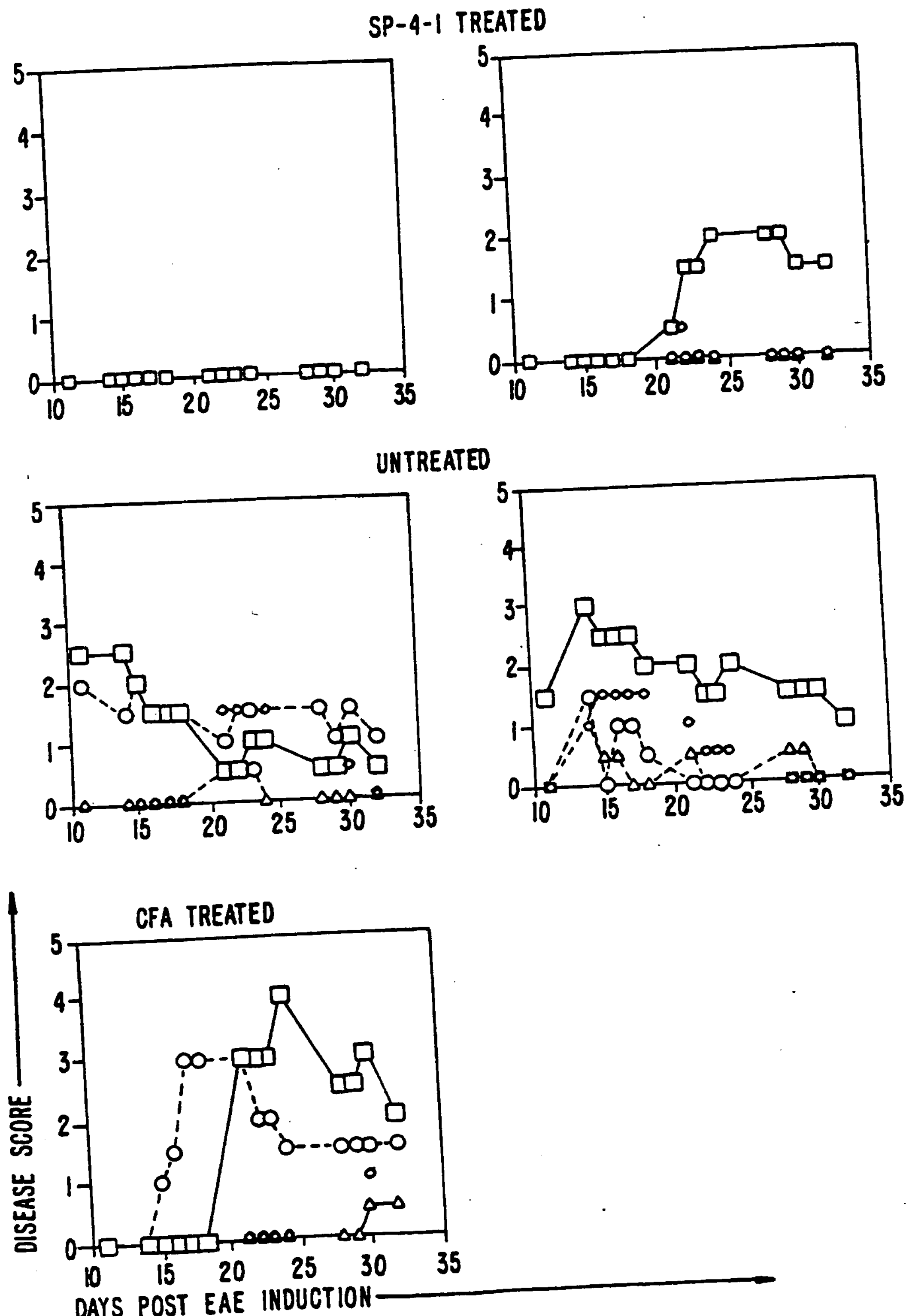

SP-4-1 TREATED
UNTREATED
CFA TREATED
DISEASE SCORE
DAYS POST EAE INDUCTION
0
1
2
3
4
5
10
15
20
25
30
35

FIG. 4.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22992

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 39/00, 38/19; C07K 14/705
US CL :424/184.1, 185.1, 198.1, 85.1; 530/351
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1, 198.1, 85.1; 530/351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West US Patent full, STN via medline, caplus, embase. Search terms: chemokine receptors, CXCR3, peptide vaccines, extracellular domain and immun?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	QIN et al. The chemokine Receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. Journal Of Clinical Investigations. February 1998, Vol. 101, No. 4, pages 746-754, especially abstract, page 750, columns 1 and 2, page 751 column 2, and figure 5A-D on page 752.	1, 10, 18 — 2-9, 11-17, 19-24
Y — A	DISIS et al. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, an oncogenic self-protein. Journal of Immunology. 1996, Vol. 156, No. 9, pages 3151-3158, especially abstract and page 3151, column 2, second paragraph.	1, 10, 18 — 2-9, 11-17, 19-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 18 NOVEMBER 1999	Date of mailing of the international search report 01 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FOZIA HAMUD Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22992

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WESTMORELAND et al. Chemokine receptor expression on resident and inflammatory cells in the brain of macaques with simian immunodeficiency virus encephalitis. American Journal of Pathology. March 1998, Vol. 152, No. 3, pages 659-665, see entire reference.	1-24
A	BONECCHI et al. Differential expression of chemokine receptors and chemotactic responsiveness of type I T helper cells (Th1s) and Th2s. Journal of Experimental Medicine. 05 January 1998. Vol. 187, No. 1, pages 129-134, see entire reference.	1-24